Remarks

I. Statement of Substance of the Interview

Further to the Interview Summary provided to Applicants' undersigned representative on September 19, 2007, Applicants submit the following Statement of the Substance of the Interview.

Applicants thank Examiner Paras and Examiner Chen for the personal interview conducted on September 19, 2007. During the interview, claims 1-45 of the present application, and the meaning of the term "non-covalently bound," were discussed. It was noted by Applicants' representatives that the presently claimed invention is directed to a virus-ligand complex, and a method of producing such a complex, where a ligand is non-covalently bound directly to a virus. Applicants' Representatives and the Examiners agreed that the term "non-covalently bound" does not mean "*not covalently bound*," and instead refers to several types of defined interactions that occur between macromolecules.

The Examiner's rejection of claims 11, 32, and 56 under 35 U.S.C. §112 was also discussed. All parties agreed that the specification and claims would be amended to include the full names for the terms EGF, VEGF, FGF and IGF, and that these amendments would not introduce new matter as these terms and their abbreviations are well-known by persons of ordinary skill in the art at the time of filing of the present application.

No further agreements were reached.

II. Status of the Claims and Support for the Amendments to the Specification and Claims

The amendments to the specification are sought to introduce the full-length names of the abbreviations EGF, VEGF, FGF and IGF. These amendments do not introduce new matter, as the meanings of the abbreviations EGF, VEGF, FGF and IGF were well known to a person of ordinary skill in the art at the time of filing of the present application, as acknowledged during the Examiner interview conducted on September 19, 2007 (*see* above). *See, e.g.*, Alberts *et al.*, Molecular Biology of the Cell, 3rd Ed., Garland Publishing Inc., New York, 1994, at page 760 (copy attached herewith as Exhibit A).

By the foregoing amendments, claims 1-18, 32-36 and 45 are sought to be amended, and claims 62-65 are sought to be added. Claims 19-31, 37-44, and 46-61 have been canceled without prejudice or disclaimer. Applicants reserve the right to pursue the subject matter of the cancelled claims in one or more divisional or continuation applications. Support for the amendments to claims 1-18, 32-36 and 45, and for new claims 62-65 can be found throughout the specification, specifically at page 9, paragraph [0036]; at page 11, paragraph [0043]; and at page 15, [0057]. Applicants note that at page 9, paragraph [0036], the present specification states that "a complex is formed between the ligand and the virus particle." Paragraphs [0043] and [0057] recite "Tf-adenovirus complexes" and "transferrin-AdLacZ complex," respectively. Therefore, Applicants respectfully submit that there is clearly support for the term "virus-ligand complex" in the present specification.

Support for the term "cell-free" recited in the amendments to claims 17 and 18 can be found throughout the specification, particularly in Example 1A at paragraph [0050]; Example 2A at paragraph [0057]; and Example 6 at paragraph [0071]. Although the term "cell-free" is not expressly stated, Applicants respectfully submit that a person of ordinary skill in the art would readily understand that preparation of the virus-ligand complex was carried out in solutions in the absence of cells, *i.e.*, "cell-free."

Claims 1-18, 32-36, 45 and 62-65 are pending in the application. Claims 19-31, 37-44 and 46-61 have been cancelled, and claims 33 and 35 have been withdrawn by the Examiner. Claims 1, 17, 62 and 64 are the independent claims.

III. Summary of the Office Action

In the Office Action dated April 30, 2007, the Examiner has made one objection to, and eight rejections of, the claims. In view of the following remarks, Applicants respectfully traverse the Examiner's objections and rejections and respectfully request that they be reconsidered and withdrawn.

IV. Claim Rejections

A. Double Patenting

In the Office Action at pages 3-7, the Examiner has provisionally rejected claims 46-61 under 37 C.F.R. § 1.75. The Examiner has stated that "should claim 1 (or claims 2-16) be found allowable, claim 46 (or claims 47-61) will be objected to

under 37 CFR § 1.75 as being a substantial duplicate thereof." *See* Office Action at pages 3-7. Applicants respectfully disagree with the Examiner and submit that claims 46-61 are not substantial duplicates of claims 1-16, and hence, do not constitute double patenting. However, solely to expedite prosecution, and not in acquiescence to this objection, claims 46-61 have been canceled without prejudice or disclaimer. Hence, the Examiner's rejection of claims 46-61 has been rendered moot.

B. Rejections under 35 U.S.C. § 112

In the Office Action at page 7, the Examiner has rejected claims 11, 32 and 56 under 35 U.S.C. §112, second paragraph, for allegedly failing to particularly point out and distinctly claim the subject matter which applicants regard as the invention. The Examiner has stated that the terms EGF, VEGF, FGF and IGF in claims 11 and 56, and the term EGF in claim 32, are allegedly vague and render the claims indefinite. The Examiner further states that these terms are abbreviations and can allegedly stand for different meanings. Applicants respectfully traverse this rejection.

Applicants submit that the meanings of these abbreviations were well-known to those of ordinary skill in the art at the time of filing of the present application, as agreed upon during the Examiner interview. *See, e.g.*, Alberts *et al.*, Molecular Biology of the Cell, 3rd Ed., Garland Publishing Inc., New York, 1994, at page 760 (Exhibit A). However, solely to expedite prosecution, Applicants have inserted the full-length names for the terms EGF, VEGF, FGF and IGF into the claims and the specification. Therefore, Applicants submit that the Examiner's rejection of claims

11, 32 and 56 has been accommodated. Reconsideration and withdrawal of this rejection are respectfully requested.

C. Rejections under 35 U.S.C. § 102

1. The Rejection Under 35 U.S.C. § 102(a) over Sosnowski

In the Office Action at pages 8-9, the Examiner has rejected claims 1-6, 8-11, 17-18, 32, 34, 45-51 and 53-56 under 35 U.S.C. § 102 (a), as allegedly being anticipated by Sosnowski *et al.*, WO 98/40508 (hereinafter "Sosnowski"). By the foregoing amendments, claims 46-51 and 53-56 have been cancelled. Hence, this rejection has been rendered moot as it may have applied to these claims. Applicants respectfully traverse this rejection as it may apply to the remaining claims.

In the Office Action at page 8, the Examiner states that:

> Sosnowski teaches preparation of a tropism-modified adenoviral vector specifically binds to target cells expressing a preselected receptor, comprising an antibody or fragment thereof that binds an adenoviral capsid protein, a targeting ligand that binds the preselected receptor, such as a polypeptide reactive with an FGF receptor, and an adenovirus containing a nucleic acid molecule encoding a therapeutic gene product under the control of a promoter, wherein the ligand is conjugated to the antibody or fragment thereof and wherein the antibody or fragment thereof is bound to the adenovirus (e.g. p. 171).

Office Action at page 8, 3rd paragraph. The Examiner concludes that "[s]ince the ligand is conjugated to the antibody or fragment thereof and the antibody or fragment thereof is bound to the adenovirus, therefore, the ligand is non-covalently bound to the adenovirus." Applicants respectfully disagree with the Examiner's contentions and conclusions.

Claim 1 (and hence, claims 2-6, 8-11, 17-18, 32 and 34 that depend ultimately therefrom) of the presently claimed invention recites a virus-ligand complex comprising a cell-targeting ligand non-covalently bound directly to a virus. Applicants submit that Sosnowski does not disclose such complexes. For example, page 26 of Sosnowski discloses a bi-specific antibody which is specifically designed to link a separate targeting moiety (a ligand) on one end to a viral capsid protein on the other end. This bi-specific antibody is not a ligand, it is a linker. (*See* Sosnowski at page 171, claim 1, ". . . wherein the ligand is conjugated to the antibody or fragment thereof and wherein the antibody or fragment thereof is bound to the adenovirus."). The ligand is separately attached to the bi-specific antibody linker and therefore is *not* non-covalently bound *directly* to the virus. Thus, there is no direct bond between the ligand and the virus in Sosnowski, much less a non-covalent bond, as required by the presently claimed invention. Furthermore, there is no linker molecule of any type in the presently claimed virus-ligand complexes, as required by Sosnowski.

Present claim 17 (and hence, claims 18 and 45 that depend ultimately therefrom) recites a method for preparing a vector virus-ligand complex comprising a cell-targeting ligand non-covalently bound directly to a virus. The method comprises mixing a cell-targeting ligand with a virus in an a cell-free aqueous medium, whereby the ligand non-covalently binds directly to the virus. As set forth above, the complexes of Sosnowski utilize a linker molecule such that the cell-targeting ligand is not non-covalently bound directly to a virus. Thus, Sosnowski does not disclose a method of preparing the virus-ligand complexes of the presently claimed invention.

Under 35 U.S.C. § 102, a claim can only be anticipated if every element in the claim is expressly or inherently disclosed in a single prior art reference. *See Kalman v. Kimberly Clark Corp.*, 713 F.2d 760, 711 (Fed.Cir. 1983), *cert. denied*, 465 U.S. 1026 (1984). Therefore, as Sosnowski does not disclose all of the elements of the present claims, specifically a virus-ligand complex comprising a cell-targeting ligand non-covalently bound directly to a virus, it cannot and does not anticipate the presently claimed invention. *Id.*

In view of the foregoing remarks, Applicants respectfully request reconsideration and withdrawal of the rejection of claims 1-6, 8-11, 17-18, 32, 34, 45-51 and 53-56 under 35 U.S.C. § 102(a).

2. The Rejection Under 35 U.S.C. § 102(b) over Satyamoorthy

In the Office Action at pages 9-10, the Examiner has rejected claims 1-4, 6, 8-11, 17-18, 45-49, 51 and 54-56 under 35 U.S.C. § 102(b), as allegedly being anticipated by Satyamoorthy *et al.*, *Cancer Research 57*:1873-1876 (1997) (hereinafter "Satyamoorthy"). By the foregoing amendments, claims 46-49, 51 and 54-56 have been cancelled. Hence, this rejection has been rendered moot as it may have applied to these claims. Applicants respectfully traverse this rejection as it may apply to the remaining claims.

In the Office Action at page 9, the Examiner states that:

> Satyamoorthy teaches mixing a recombinant bFGF-SAP (basic fibroblast growth factor-plant toxin saporin) with a replication-defective adenovirus Ad5 containing LacZ reporter gene and shows that infection of melanoma cells with a replication-defective

> adenovirus enhances cell killing by a mitotoxin bFGF-SAP more than 10-fold (e.g. abstract, right column under Materials and Methods).

Office Action at page 9, 3rd full paragraph. The Examiner concludes that the presently claimed invention is allegedly anticipated as "[a] replication deficient adenovirus is considered a recombinant virus" and "[t]he bFGF-SAP is a recombinant protein." Office Action at page 10, lines 1-3. Applicants respectfully disagree with the Examiner's conclusions and contentions.

As set forth above, claim 1 of the presently claimed invention recites virus-ligand complex comprising a cell-targeting ligand non-covalently bound directly to a virus. Satyamoorthy discloses transfecting melanoma cell lines with either bFGF (a cell-targeting ligand), saporin (SAP; a plant mitotoxin used to induced apoptosis), a bFGF-SAP conjugate, a replication-defective adenovirus (Ad5) containing a LacZ reporter gene, or mixtures thereof. While bFGF and SAP may be present in the complexes of Satyamoorthy, the Ad5 virus is administered to, and enters, cells separately from the bFGF-SAP complex. *See* Satyamoorthy at p. 1873, 2nd column, Results, lines 6-9. The cell-targeting ligand is therefore *not* non-covalently bound directly to the virus as required by the presently claimed invention. In fact, it is not bound to the virus at all.

Satyamoorthy states that "cells were treated with SAP, bFGF-SAP, bFGF, or Ad5 alone or in combination." (Satyamoorthy at p. 1873, 2nd column, lines 10-11 under Materials and Methods). Satyamoorthy also discloses "combining bFGF-SAP treatment of cells with Ad5 infection." (*See* Satyamoorthy at p. 1873, Results, lines 14-16). However, Satyamoorthy does not disclose that the bFGF-SAP and the Ad5

exist in a single complex where the bFGF-SAP complex (or either ligand separately) is *non-covalently bound directly* to the virus. In fact, formation of a complex between the bFGF-SAP and the Ad5 virus would have been contrary to the purpose of Satyamoorthy, which was to utilize the synergistic (*i.e.*, separately administered) interaction between the ligand and the virus. (*See* Satyamoorthy at page 1873, Introduction, lines 22-24.) Therefore, Satyamoorthy does not disclose all of the elements of the present claims, and hence it cannot anticipate the presently claimed invention. *See Kalman*, 713 F.2d at 711.

Present claim 17 (and hence, claims 18 and 45 that depend ultimately therefrom) recites a method for preparing a vector virus-ligand complex comprising a cell-targeting ligand non-covalently bound directly to a virus. The method comprises mixing a cell-targeting ligand with a virus in an a cell-free aqueous medium, whereby the ligand non-covalently binds directly to the virus.

As noted above, the bFGF-SAP complex and the adenovirus disclosed in Satyamoorthy are administered to MCDB153/L15 cells separately. (*See* Satyamoorthy at page 1873, 2nd column, lines 10-11). Therefore, even assuming *arguendo* that a complex is ever formed between the bFGF-SAP conjugate and the adenovirus (which would be contrary to the purpose of Satyamoorthy), it would only form in the presence of cells. The presently claimed methods recite that the cell-targeting ligand is *non-covalently* bound *directly* to a virus, and that the virus-ligand complex is prepared by mixing the cell-targeting ligand with the virus in a *cell-free* aqueous medium. Satyamoorthy does not disclose either of these requirements of the methods of the presently claimed invention. Therefore, Satyamoorthy does not disclose all of

the elements of the present claims, and hence it cannot anticipate the presently claimed invention. *See Kalman*, 713 F.2d at 711.

In view of the foregoing remarks, Applicants respectfully request reconsideration and withdrawal of the rejection of claims 1-4, 6, 8-11, 17-18, 45-49, 51 and 54-56 under 35 U.S.C. § 102(b).

3. The Rejection Under 35 U.S.C. § 102(b) over Cotten

In the Office Action at pages 10-11, the Examiner has rejected claims 1-2, 6, 8-10, 12, 15-18, 32, 45-47, 51, 53-55, 57 and 60-61 under 35 U.S.C. § 102(b), as allegedly being anticipated by Cotten *et al.*, *Proc Natl Acad Sci USA 89*: 6094-6098 (1992) (hereinafter "Cotten"). By the foregoing amendments, claims 46-47, 51, 53-55, 57 and 60-61 have been cancelled. Hence, this rejection has been rendered moot as it may have applied to these claims. Applicants respectfully traverse this rejection as it may apply to the remaining claims.

In the Office Action at page 10, the Examiner states that:

> Cotten teaches a plasmid DNA expressing luciferase under the control of RSV promoter with human or mouse transferrin-polylysine (equivalent to 4 μg polylysine) and the mixed solution is further mixed with replication-defective human adenovirus 5 lacking functional E1a sequence (e.g. p. 6095, left column, 3rd paragraph).

Office Action at page 10, last paragraph. The Examiner further states that "the transferrin . . . is not covalently bound to the adenovirus." Office Action at page 11, lines 3-5. The Examiner therefore concludes that the presently claimed invention is

allegedly anticipated. Applicants respectfully disagree with the Examiner's contentions and conclusions.

As set forth above, present claim 1 (and hence, claims 2-6, 8-11, 17-18, 32 and 34 that depend ultimately therefrom) recites a virus-ligand complex comprising a cell-targeting ligand non-covalently bound directly to a virus. As discussed during the Examiner interview, a "non-covalent bond" refers to a class of specific molecular interactions. It does *not* mean that the cell-targeting ligand and the virus are "not covalently bound" to one another.

Cotten discloses treating mammalian cell lines with a mouse transferrin-polylysine complex and an adenovirus containing a β-galactosidase reporter gene. The transferrin acts as a cell-targeting ligand in this case. While the transferrin and the polylysine are present in a complex, the adenovirus is administered to and enters the cells *separately* from the transferrin-polylysine complex. *See* Cotten at page 6905, Figure 1. Cotten does not disclose that the ligand is non-covalently bound directly to the virus as required by the presently claimed invention. In fact, there is no bond between the ligand and the virus. The Examiner's statement that "the transferrin . . . is not covalently bound to the adenovirus," is in fact correct. Office Action at page 11, lines 3-5. However, this clearly does not anticipate the presently claimed virus-ligand complexes which require a *non-covalent bond directly* between the cell-targeting ligand and the virus. Furthermore, formation of a bond between transferrin and the adenovirus in Cotten would defeat the purpose of Cotten, which requires separate binding of the ligand and the virus to the cell surface. *See* Cotten at page 6095, Figure 1.

Therefore, Cotten does not disclose each and every element of the presently claimed invention, specifically a virus-ligand complex comprising a cell-targeting ligand non-covalently bound directly to a virus. Hence, Cotten cannot and does not anticipate the presently claimed invention. *See Kalman*, 713 F.2d at 711.

Cotten also does not disclose the methods of the presently claimed invention for preparing the virus-ligand complexes. As noted above, the transferrin-polylysine conjugate and the adenovirus particles of Cotten are administered separately to cells (*See* Cotten at p. 6095, Figure 1, legend). Therefore, even assuming *arguendo* that a complex is ever formed between the transferrin-polylysine conjugate and the adenovirus (which would be contrary to the purpose of Cotten), it would form in the *presence* of cells. The ligand (transferrin) and the virus are not mixed in a cell-free aqueous medium in Cotten to form a virus-ligand complex, as required by the presently claimed method. Therefore, as Cotten does not disclose all of the elements of the present claims, it cannot and does not anticipate the presently claimed invention. *See Kalman*, 713 F.2d at 711.

In view of the foregoing remarks, Applicants respectfully request reconsideration and withdrawal of the rejection of claims 1-2, 6, 8-10, 12, 15-18, 32, 45-47, 51, 53-55, 57 and 60-61 under 35 U.S.C. § 102(b).

4. The Rejection Under 35 U.S.C. § 102(b) over Seth

In the Office Action at pages 11-12, the Examiner has rejected claims 1-2, 6, 9-11, 15-18, 32, 45-47, 51, 54-56 and 60-61 under 35 U.S.C. § 102(b), as allegedly being anticipated by Seth *et al.*, *Journal of Virology 51*:650-655 (1984) (hereinafter

"Seth"). By the foregoing amendments, claims 46-47, 51, 54-56 and 60-61 have been cancelled. Hence, this rejection has been rendered moot as it may have applied to these claims. Applicants respectfully traverse this rejection as it may apply to the remaining claims.

In the Office Action at page 12, the Examiner states that "Seth teaches preparation of recombinant PE-EGF (*Pseudomonas* exotoxin and epidermal growth factor) hybrid toxin and mixing adenovirus and PE-EGF in 1.5 mL of fresh medium." Office Action at page 12, lines 3-4. The Examiner further states that "EGF is a cell-targeting ligand and the PE-EGF hybrid toxin is not covalently bound to the adenovirus." Office Action at page 12, lines 13-14. The Examiner therefore concludes that Seth allegedly anticipates the presently claimed invention. Applicants respectfully disagree with the Examiner's contentions and conclusions.

As set forth above, the presently claimed invention recites a virus-ligand complex comprising a cell-targeting ligand non-covalently bound directly to a virus, as well as methods for forming such complexes by mixing the recited components in a cell-free aqueous medium. Applicants respectfully submit that Seth does not disclose a virus-ligand complex where a cell-targeting ligand is non-covalently bound directly to a virus. In fact, there is no bond between the ligand and the virus disclosed in Seth.

Seth discloses treating KB cells with a *Pseudomonas* exotoxin-EGF conjugate and adenovirus separately. The EGF acts as a cell-targeting ligand. The exotoxin-EGF and adenovirus are administered to KB cells separately. (*See* Seth at page 650, 2nd column, ll. 12-14). Thus, as noted by the Examiner, "the PE-EGF hybrid toxin is not covalently bound to the adenovirus," as there is *no bond at all* between the PE-

EGF and the adenovirus. Therefore, clearly there cannot be *non-covalent bond* between the ligand (PE-EGF in Seth) and the adenovirus, as required by the presently claimed invention.

Furthermore, even assuming *arguendo* that a complex is formed between the exotoxin-EGF conjugate and the adenovirus, it is formed in the presence of cells. Thus, any exotoxin-EGF-adenovirus complex (which would be contrary to the purpose of Seth), is not formed in a cell-free aqueous medium as required by the method claims of the present invention. Therefore, as Seth does not disclose all of the elements of the present claims, it cannot and does not anticipate the presently claimed invention. *See Kalman*, 713 F.2d at 711.

In view of the foregoing remarks, Applicants respectfully request reconsideration and withdrawal of the rejection of claims 1-2, 6, 9-11, 15-18, 32, 45-47, 51, 54-56 and 60-61 under 35 U.S.C. § 102(b).

5. The Rejection Under 35 U.S.C. § 102(b) over Spooner

Claims 1-12, 17-18, 32, 34 and 45-47 were rejected under 35 U.S.C. §102(b), as allegedly being anticipated by Spooner *et al.*, WO 94/10323 (1994) (hereinafter "Spooner"). By the foregoing amendments, claims 46 and 47 have been cancelled. Hence, this rejection has been rendered moot as it may have applied to these claims. Applicants respectfully traverse this rejection as it may apply to the remaining claims.

In the Office Action at page 13, the Examiner states that:

> Spooner teaches a preparation of a virus or virus-like particle, such as vaccinia virus, adenovirus, any other animal virus or replication-defective derivative thereof, having a modified binding specificity

> conferred by a binding moiety, such as a ligand of a target cell-specific cell-surface receptor, allowing the virus or virus-like particle to bind to a target cell (e.g. p. 77) . . . . The binding moiety may be linked to the polypeptide on the surface of the virus or virus-like particle by any of the conventional ways of cross-linking polypeptides (e.g. p. 23, first paragraph).

Office Action at page 13, 2nd paragraph. The Examiner concludes that "the binding moiety (ligand) can be non-covalently bound to the virus or virus-like particle." *See* Office Action at page 13, lines 21-22. Applicants respectfully disagree with the Examiner's contentions and conclusions.

As discussed above, the presently claimed invention recites a virus-ligand complex comprising a cell-targeting ligand non-covalently bound directly to a virus, as well as methods for forming such complexes by mixing the recited components in a cell-free aqueous medium.

Applicants respectfully submit that Spooner only discloses chemical conjugation of a binding moiety to a polypeptide on the surface of the virus, and not formation of a non-covalent bond between a cell-targeting ligand and a virus as required by the presently claimed invention. For example, Spooner states that "[i]f the binding moiety and the molecule on the surface of the virus or virus-like particle are both polypeptides, then they may be linked together by any of the conventional ways of cross-linking polypeptides... [f]or example, the binding moiety may be enriched with thiol groups and the molecule on the surface of the virus or virus-like particle reacted with a bifunctional agent capable of reacting with those thiol groups." (*See* Spooner at page 23, lines 1-12). Each of the methods discussed in Spooner involves chemical conjugation, *not* non-covalent bonding. Spooner states that

"[o]ther chemical procedures may be useful in joining oligosaccharide and lipids to other oligosaccharides, lipids or polypeptides." (*See* Spooner at page 23, lines 14-15). The reference cited by Spooner on page 23, lines 9-10 (O'Sullivan *et al.*, *Anal. Biochem. 100*: 100-108 (1979); copy attached hereto as Exhibit B) discloses only covalent conjugation via two different chemical methods. (*See* O'Sullivan, at Abstract: "Sulfhydryl residues were introduced into second antibodies . . . . Up to 80% of the enzyme was conjugated to immunologically active antibody . . . ."). Spooner also references thiol groups, which are used for chemical (i.e., covalent) conjugation.

Thus, all of the methods of ligand attachment disclosed in Spooner involve chemical conjugation via covalent bonding, and do *not involve non-covalent bonding*. Therefore, Spooner does not disclose a virus-ligand complex comprising a cell-targeting ligand non-covalently bound directly to a virus, or a method for producing such a virus-ligand complex, as recited by the presently claimed invention. Hence, Spooner does not disclose all of the elements of the present claims, and therefore this reference cannot and does not anticipate the presently claimed invention. *See Kalman*, 713 F.2d at 711.

In view of the foregoing remarks, Applicants respectfully request reconsideration and withdrawal of the rejection of claims 1-12, 17-18, 32, 34 and 45-47 under 35 U.S.C. § 102(b).

D. Rejections under 35 U.S.C. § 103

1. The Rejection Under 35 U.S.C. § 103(a) over Cotten or Seth

Claims 1, 13-14, 46, 58 and 59 were rejected under 35 U.S.C. §103(a) as allegedly being unpatentable over either Cotten or Seth. By the foregoing amendments, claims 46, 58 and 59 have been cancelled. Hence, this rejection has been rendered moot as it may have applied to these claims. Applicants respectfully traverse this rejection as it may apply to the remaining claims.

In the Office Action at page 15, the Examiner states that

> [i]t would have been obvious for one of ordinary skill in the art at the time of the invention to use the ratio of the cell-targeting ligand and the virus in the range of 100 to 1,000,000 or 6700 to 4,000,000 ligand molecules per virion because Cotten teaches mixing the mouse transferrin-polylysine (mTfpL) with 10 μL of adenovirus containing $5x10^{11}$ particles/mL, and Seth teaches mixing adenovirus and PE-EGF in 1.5 mL of fresh medium and the concentration of adenovirus is 0.1 to 10 μg/mL and the concentration of PE-EGF is 0.01 to 0.5 μg/mL . . . .

Office Action at page 15, 1st paragraph. The Examiner further states that:

> it would be obvious for one of ordinary skill in the art at the time the invention was made to use the claimed range of ratio of adenovirus and ligand in order to provide better protein synthesis in KB cells by PE-EGF as taught by Seth or to provide high-efficiency receptor-mediated gene delivery via the use of defective adenovirus particles as taught by Cotten with reasonable expectation of success.

Office Action at page 15, lines 14-18. The Examiner therefore concludes that the presently claimed invention is rendered obvious. Applicants respectfully disagree with the Examiner's contentions and conclusions.

As discussed above, Cotten discloses treating mammalian cell lines with a mouse transferrin-polylysine complex and an adenovirus containing a β-galactosidase reporter gene. The transferrin acts as a cell-targeting ligand. While the transferrin and the polylysine are present in a complex, the adenovirus is administered to, and enters, cells *separately* from the transferrin-polylysine complex. *See* Cotten at page 6095, Figure 1. Hence, Cotten does not disclose that the ligand is non-covalently bound directly to the virus as required by the presently claimed invention. In fact, there is no bond between the ligand and the virus. Furthermore, a bond between the ligand and the virus would defeat the purpose of Cotten which requires separate binding of the ligand and the virus to the cell. *See* Cotten, Figure 1.

As discussed above, Seth discloses treating KB cells with a *Pseudomonas* exotoxin-EGF conjugate and adenovirus separately. The EGF acts as a cell-targeting ligand in this case. The exotoxin-EGF and adenovirus must be administered to KB cells separately in order to function as required by Seth (*See* Seth at page 650, 2nd column, lines 12-14). Hence, Seth does not disclose that the ligand is non-covalently bound directly to the virus as required by the presently claimed invention. In fact, there is no bond between the ligand and the virus. Furthermore, a bond between the ligand and the virus would defeat the purpose of Seth, which requires separate binding of the ligand and the virus to the cell.

"Under § 103, the scope and content of the prior art is to be determined; differences between the prior art and the claims at issue are to be ascertained; and the level of ordinary skill in the pertinent art resolved. Against this background the obviousness or nonobviousness of the subject matter is determined." *Graham v. John*

Deere Co. of Kansas City, 383 U.S. 1, 17 (1966). Applicants respectfully submit that the differences between the presently claimed invention and the reference cited by the Examiner are so great that it would not have been obvious to modify the disclosures of Cotten or Seth, as required by the Examiner, in order to render the presently claimed invention obvious.

As set forth above, Applicants respectfully submit that neither Cotten nor Seth disclose a virus-ligand complex comprising a cell-targeting ligand non-covalently bound directly to a virus. Furthermore, formation of such a virus-ligand complex would have rendered the disclosures of Cotten and Seth inoperable for their intended purposes, as both references require the *separate* administration of the cell-targeting ligand and the virus. *See* M.P.E.P. § 2143.01(V) "If proposed modification would render the prior art invention being modified unsatisfactory for its intended purpose, then there is no suggestion or motivation to make the proposed modification. *In re Gordon*, 733 F.2d 900, 221 USPQ 1125 (Fed. Cir. 1984)." Applicants therefore submit that Seth and Cotten do not render obvious the presently claimed invention. In view of the foregoing remarks, Applicants respectfully request reconsideration and withdrawal of this rejection.

2. The Rejection Under 35 U.S.C. § 103(a) over Spooner in view of Kingsman

In the Office Action at pages 15-16, the Examiner has rejected claims 1 and 36 under 35 U.S.C. §103(a), as allegedly being unpatentable over Spooner in view of

Kingsman *et al.*, WO 97/32026 (1997) (hereinafter "Kingsman"). Applicants respectfully traverse this rejection.

In the Office Action at page 16, the Examiner states that

> [i]t would have been obvious for one of ordinary skill in the art at the time of the invention to use retrovirus having a transferrin as a ligand for gene delivery to target cells because Spooner teaches using adenovirus or vaccinia virus having a transferrin as a ligand for gene delivery in gene therapy and Kingsman teaches using a retrovirus for gene delivery in gene therapy.

Office Action at page 16, 4th paragraph. The Examiner concludes that "[o]ne having ordinary skill in the art at the time the invention was made would have been motivated to [combine the teachings of Spooner and Kingsman] in order to deliver gene to target cells in gene therapy as taught by Spooner and Kingsman with reasonable expectation of success." Office Action at page 16, 5th paragraph. Applicants respectfully disagree with the Examiner's conclusions and contentions.

Applicants respectfully submit that neither Spooner nor Kingsman discloses a virus-ligand complex comprising a cell-targeting ligand non-covalently bound directly to a virus, as required by the presently claimed invention.

As discussed above, each of the methods disclosed in Spooner involves chemical conjugation resulting in covalent bonding, not non-covalent bonding between virus and ligand. Thus, as discussed above, Spooner does not disclose a cell-targeting ligand non-covalently bound directly to a virus. Furthermore, it would not have been obvious to even try and prepare a virus-ligand complex comprising a cell-targeting ligand non-covalently bound directly to a virus based on the disclosure of

Spooner, as Spooner clearly relies on chemical conjugation of the binding moiety to the virus to form the disclosed stable complexes. (*See* Spooner at page 23, lines 1-12).

Kingsman discloses that the "adapter molecules described herein would be administered to the target cells either *in vivo* or *in vitro,* just prior to the administration of corresponding ecotropic vector particles." (*See* Kingsman at page 4, line 26-28.) Thus, any complex formed between an adapter molecule (which serves as the ligand in this case) and a virus particle, in Kingsman would only be formed in the presence of cells, and only after the adapter molecule is attached to the target cell. Therefore, Kingsman does not disclose preparation of a ligand-virus complex external from a cell, "wherein upon introduction of said virus ligand complex into a host animal, said ligand binds directly to a receptor on said target cell," as required by present claim 1. In fact, formation of a virus-ligand complex *outside of a cell*, which, then upon introduction into a host animal binds directly to a receptor on a target cell, would have been contrary to the purpose of Kingsman, which requires the use of "unmodified ectropic vectors to deliver genes to [] cells." Kingsman at page 2, line 26. Preparation of a virus-ligand complex, a "modified vector," as required by the presently claimed invention, would clearly render Kingsman unsatisfactory for its intended purpose. *See* M.P.E.P. § 2143.01(V).

Therefore, Applicants respectfully submit that as neither Spooner nor Kingsman disclose or suggest a virus-ligand complex comprising a cell-targeting ligand non-covalently directly bound to a virus, which, when introduced into a host animal, binds directly to a receptor on a target cell, the combination of these references clearly cannot render obvious the presently claimed invention. As noted

above, all of the conjugation methods disclosed in Spooner require covalent bonding, and all of the complexes disclosed in Kingsman require formation on the surface of a cell. Therefore, it would not have been obvious to prepare, or to even try and prepare, the virus-ligand complexes of the presently claimed invention where a cell-targeting ligand is non-covalently bound directly to a virus.

Applicants therefore submit that Spooner and Kingsman, alone or in combination, do not render obvious the presently claimed invention. In view of the foregoing remarks, Applicants respectfully request reconsideration and withdrawal of the rejection of claims 1 and 36 under 35 U.S.C. § 103(a).

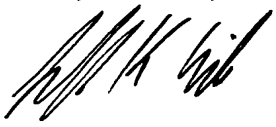
Conclusion

All of the stated grounds of rejection have been properly traversed, accommodated or rendered moot. Applicants therefore respectfully request that the Examiner reconsider all presently outstanding rejections and that they be withdrawn. Applicants believe that a full and complete reply has been made to the outstanding Office Action and, as such, the present application is in condition for allowance. If the Examiner believes, for any reason, that personal communication will expedite prosecution of this application, the Examiner is invited to telephone the undersigned at the number provided.

Prompt and favorable consideration of this Amendment and Reply is respectfully requested.

Respectfully submitted,

STERNE, KESSLER, GOLDSTEIN & FOX P.L.L.C.



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Date: October 1, 2007

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Exhibit

A

MOLECULAR BIOLOGY OF
THE CELL
THIRD EDITION

Bruce Alberts • Dennis Bray
Julian Lewis • Martin Raff • Keith Roberts
James D. Watson

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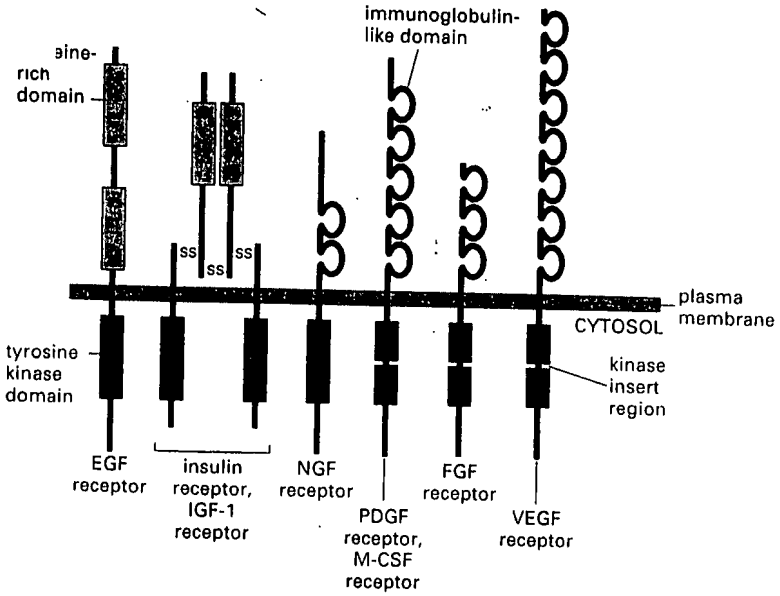

Figure 15–47 Six subfamilies of receptor tyrosine kinases. Only one or two members of each subfamily are indicated. Note that the tyrosine kinase domain is interrupted by a "kinase insert region" in some of the subfamilies. The functional significance of the cysteine-rich and immunoglobulinlike domains is unknown.

The Receptors for Most Growth Factors Are Transmembrane Tyrosine-specific Protein Kinases [32]

The first receptor protein recognized to be a **tyrosine-specific protein kinase** (in 1982) was the receptor for *epidermal growth factor (EGF)*. EGF is a small protein (53 amino acids) that stimulates the proliferation of epidermal cells and a variety of other cell types. Its receptor is a single-pass transmembrane protein of about 1200 amino acids, with a large glycosylated extracellular portion that binds EGF. An intracellular tyrosine kinase domain is activated when EGF binds to the receptor. Once activated, the receptors transfer a phosphate group from ATP to selected tyrosine side chains, both on the receptor proteins themselves and on specific cellular proteins. Many other receptors for growth and differentiation factors are also **receptor tyrosine kinases.** These include the receptors for *platelet-derived growth factor (PDGF), fibroblast growth factors (FGFs), hepatocyte growth factor (HGF), insulin, insulinlike growth factor-1 (IGF-1), nerve growth factor (NGF), vascular endothelial growth factor (VEGF),* and *macrophage colony stimulating factor (M-CSF),* all of which are proteins. As shown in Figure 15–47, the family of receptor tyrosine kinases can be divided into a number of structural subfamilies; in each case the receptors phosphorylate themselves to initiate the intracellular signaling cascade.

How does the binding of a specific protein to the extracellular portion of a receptor tyrosine kinase activate the catalytic domain on the other side of the plasma membrane? It is difficult to imagine how a conformational change could propagate across the lipid bilayer through a single transmembrane α helix. The puzzle was solved when it was demonstrated that ligand binding causes the EGF receptor to assemble into dimers, which enables the two cytoplasmic domains to cross-phosphorylate each other on multiple tyrosine residues. This cross-phosphorylation is referred to as *autophosphorylation* because it occurs within the receptor dimer. In the case of PDGF receptors the ligand is a dimer that cross-links two receptors together (Figure 15–48). EGF, by contrast, is a monomer that is thought to induce a conformational change in the extracellular domain of its receptors to induce receptor dimerization. It is thought that receptor dimerization is a general mechanism for activating enzyme-linked receptors with a single transmembrane domain.

Receptor dimerization can be exploited experimentally to inactivate specific receptors in order to determine their importance for a particular cell response. The strategy involves transfecting cells with DNA that encodes a mutant form of a receptor tyrosine kinase that dimerizes normally but has an inactive kinase

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Front cover: The photograph shows a rat nerve cell in culture. It is labeled (*yellow*) with a fluorescent antibody that stains its cell body and dendritic processes. Nerve terminals (*green*) from other neurons (not visible), which have made synapses on the cell, are labeled with a different antibody. (Courtesy of Olaf Mundigl and Pietro de Camilli.)

Dedication page: Gavin Borden, late president of Garland Publishing, weathered in during his mid-1980s climb near Mount McKinley with MBoC author Bruce Alberts and famous mountaineer guide Mugs Stump (1940–1992).

Back cover: The authors, in alphabetical order, crossing Abbey Road in London on their way to lunch. Much of this third edition was written in a house just around the corner. (Photograph by Richard Olivier.)

Exhibit B

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Comparison of Two Methods of Preparing Enzyme–Antibody Conjugates: Application of These Conjugates for Enzyme Immunoassay

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Two methods of preparing enzyme–antibody conjugates were evaluated. High yields of conjugate were obtained with both methods. The first procedure utilizes the homobifunctional crosslinking reagent *N*,*N*′-*o*-phenylenedimaleimide. Sulfydryl residues were introduced into second antibodies by reaction with methyl-mercaptobutyrimidate. The modified antibodies were reacted with *N*,*N*′-*o*-phenylenedimaleimide, excess reagent was removed by gel filtration, and the activated antibodies were cross-linked to β-galactosidase. Up to 80% of the enzyme was conjugated to immunologically active antibody with approximately 90% retention of enzyme activity. The second method utilizes the heterobifunctional *meta*-maleimidobenzoyl-*N*-hydroxysuccinimide ester (MBS). The antibodies were reacted with MBS, excess reagent was removed by gel filtration and the activated antibodies were crosslinked to β-galactosidase. Typically, approximately 80% of the enzyme was conjugated to immunologically active antibody with approximately 90% retention of both enzyme and antibody activity. Conjugates prepared using these two procedures were used as labels in an immunoassay system and were able to detect approximately 5 to 10 ng of first antibodies. The MBS procedure was simpler to perform, could more easily be adapted to large-scale work, and gave more reproducible results, and the conjugates produced were able to detect slightly lower concentrations of first antibody.

Enzymes have been used as labels in immunohistochemistry and in enzyme immunoassay (EIA)[2] for a number of years. These labels are generally considered to provide less assay sensitivity than radiolabels. However, several workers have recently shown that when sufficient attention is given to the choice of enzyme, crosslinking method, and the detection system, very sensitive EIAs can be devised (1–4).

The use of enzyme-labeled antibodies in EIA is particularly attractive as the same procedure can probably be generally applied to labeling different antibodies. Unfortunately, there are very few efficient general methods available for the production of such conjugates. Typically, the labels are prepared in low yield with considerable loss of both enzyme and antibody activity, and extensive polymerization resulting in high-molecular-weight conjugates often occurs. Glutaraldehyde (5) and the periodate procedure (6) are the most commonly used methods. Although the glutaraldehyde procedure is very easy to perform, typically the conjugates are prepared in low yields with loss of enzyme and antibody activity and polymerized conjugates are formed (7).

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[2] Abbreviations used: EIA, enzyme immunoassay; MBS, *meta*-maleimidobenzoyl-*N*-hydroxysuccinimide ester; T_3, triiodothyronine; EDC, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide; ONPG, *o*-nitrophenyl-β-D-galactopyranoside; BSA, bovine serum albumin; PAG, polyacrylamide gel; HgH, human growth hormone; IgG, immunoglobulin G; RIA, radioimmunoassay; DTNB, 5,5′-dirhio-bis-2-nitrobenzoic acid.

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With the periodate method, yields of up to 70% have been reported, but this method is limited to glycoprotein enzymes.

Recently, *N,N'-o*-phenylenedimaleimide has been used to conjugate β-galactosidase to antibodies (8). This reagent has the potential merit that it reacts rapidly and selectively with sulfydryl residues under mild conditions. In this paper the use of this reagent is compared with that of *meta*-maleimidobenzoyl-*N*-hydroxysuccinimide ester (MBS). MBS has previously been used to conjugate β-galactosidase to viomycin (9) and insulin (1). Due to the heterobifunctional nature of MBS and the lack of sulfydryl residues in antibodies, this reagent should not polymerize antibody molecules. A preliminary account of the use of MBS in the production of enzyme–antibody conjugates has been published (10).

MATERIALS AND METHODS

Triiodothyronine (T_3), bovine insulin, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), *o*-nitrophenyl-β-D-galactopyranoside (ONPG), β-mercaptoethanol and Sepharose 4B were purchased from the Sigma Chemical Company (St. Louis, Mo.); cellulose powder CC41 from Whatman Ltd. (Maidstone, England); 1,4-butanediol diglycidyl ether and *N,N'-o*-phenylenedimaleimide from Aldrich Chemical Company Ltd. (Gillingham, England); methyl-mercaptobutyrimidate and MBS from Pierce Chemical Company (Rockford, Ill.); bovine serum albumin (BSA) from Armour Pharmaceutical Company Ltd. (Eastbourne, England); DEAE-Bio-Gel A from Bio-Rad Laboratories (Richmond, Calif.); and Sephadex G-25 from Pharmacia Ltd. (Uppsala, Sweden).

Escherichia coli. β-D-galactosidase (EC 3.2.1.23) was purchased from Boehringer–Mannheim (Mannheim, West Germany). The preparation was composed of approximately 90% enzyme as estimated by polyacrylamide gel (PAG) electrophoresis and Sepharose gel filtration. Human growth hormone (HgH) was a gift from Dr. P. J. Lowry of St. Bartholomew's Hospital (London, England). All other chemicals were purchased from BDH Chemicals Ltd. (Poole, England).

Antiserum production. The production of antibodies to T_3 (sheep), HgH (rabbit), sheep IgG, and rabbit Fc fragments (donkeys) has been described previously (11). Cortisol (sheep) and donkey anti-guinea pig IgG antibodies were purchased from Guildhay Antisera (Guildford, England). Insulin antibodies (guinea pig) were purchased from Wellcome Reagents Ltd. (Beckenham, England).

The concentration of antibodies present in the T_3, HgH, cortisol, and insulin antisera was calculated from binding site data derived from Scatchard plots obtained by radioimmunoassay (RIA) (11). It was assumed that each antibody molecule had two antigen binding sites.

Preparation of solid phases. Immunoadsorbents for the purification of second antibodies were prepared by conjugating the immunoglobulin fraction of either sheep, rabbit, or guinea pig serum to Sepharose 4B using the crosslinking reagent butanediol diglycidyl ether (12), as previously described (11). Typically, 5 mg of protein was coupled per milliliter of swollen gel.

Solid phases for the determination of second antibody activity were prepared by coupling the immunoglobulin fraction of either sheep, rabbit, or guinea pig serum to amino-cellulose (13). Typically, 500 mg of protein was coupled per gram of amino-cellulose.

Solid phases for the detection of HgH and insulin antibodies by EIA were also prepared using amino-cellulose (13). Ninety milligrams of HgH and 150 mg of insulin were coupled per gram of amino-cellulose. The T_3 solid phase for the detection of T_3 antibodies by EIA was prepared from a cellulose suspension (13) activated with butanediol diglycidyl ether (12) and reacted with T_3 dissolved in 50 mM phosphate buffer, pH

12.0, for 24 h at room temperature. Approximately 50 μg of T_3 was conjugated per gram of cellulose.

The cortisol solid phase for the detection of cortisol antibodies by EIA was prepared by coupling cortisol hemisuccinate to an amino-cellulose suspension using the carbodiimide procedure (14). The degree of incorporation of cortisol into the solid phase was not measured. However, the treated solid phase was able to adsorb cortisol antibodies from cortisol antiserum as determined by RIA (11).

Determination of β-galactosidase and second antibody activity. Enzyme activity was measured using ONPG as substrate (15). The degree of conjugation of enzyme to immunologically active second antibody was determined by measuring the binding of the conjugate to an IgG solid phase. The enzyme–second antibody conjugate (0.1 ml) was mixed with 0.1 mg of solid phase suspended in 0.1 ml of 50 mM barbitone buffer, pH 8.6, containing 1 g/liter BSA for 4 h at 4°C. The solid phase was washed twice with 2 ml of barbitone buffer; 1 ml of substrate solution was added to the solid phase, mixed, and incubated at room temperature with constant mixing. (Later experiments were carried out at 30°C; continual mixing was found to be unnecessary.) After a suitable period (20 to 60 min) the reaction was terminated, the absorbance measured at 420 nm, and the proportion of enzyme activity bound to the solid phase determined.

The second-antibody activity was measured using a "sandwich assay" involving an immunoglobulin solid phase and ^{125}I-labeled immunoglobulin. Radioactivity bound to the solid phase was proportional to the concentration of second antibody. The details of this assay have been described (11).

Preparation of enzyme–antibody conjugates using N,N'-o-phenylenedimaleimide. One milliliter of Sepharose–sheep IgG was mixed with 3 ml of donkey anti–sheep IgG (D20 11.3.77) for 1 h at room temperature. Nonspecifically bound protein was removed by washing 10 times with 10 ml of 50 mM phosphate buffer, pH 7.0, containing 0.2 M NaCl. The gel was suspended in 9 ml of 50 mM *N*-ethylmorpholine–HCl buffer, pH 7.5, to which 0.1 ml of a 10 mg/ml solution of methyl-mercaptobutyrimidate in ice-cold 0.1 M Na_2CO_3 was added. The gel suspension was mixed for 1 h at room temperature and then washed five times with 10 ml of distilled water. The gel was packed into a 5-ml disposable syringe fitted with a glass wool filter and the gel washed with 10 ml of HCl, pH 3.5, containing 0.1 M NaCl. The modified antibodies were eluted with 5 ml of HCl, pH 2.5, into 0.5 ml of 50 mmol acetate buffer, pH 6.0. One milliliter of a saturated solution of *N,N'-o*-phenylenedimaleimide in 0.1 M acetate buffer, pH 5.0, at 30°C was added to the antibody solution, mixed, and maintained at 30°C for 30 min. To remove excess *N,N'-o*-phenylenedimaleimide, the solution was chromatographed on a Sephadex G-25 column (60 × 0.9 cm) equilibrated with 50 mM acetate, pH 5.5, containing 10 mM $MgCl_2$. Fractions containing protein (4.5 ml, 2.0 mg protein) were pooled, and 2.0 mg of β-galactosidase was added, mixed, and held at 30°C for 1 h when the reaction was terminated by the addition of 1 M β-mercaptoethanol to give a final concentration of 10 mM. The reaction mixture was dialyzed overnight at 4°C against 1 liter of 10 mM Tris–HCl buffer, pH 7.5, containing 10 mM $MgCl_2$ and 10 mM mercaptoethanol.

The number of sulfydryl residues introduced per antibody molecule by reaction with mercaptobutyrimidate was determined by carrying out this reaction as described above but eluting the modified antibodies into 0.5 ml of 0.2 M borate buffer, pH 8.6. The sulfydryl content of the antibodies was then determined by reaction with DTNB (16).

Purification of the conjugate. The dialyzed reaction mixture was applied to a DEAE Bio-Gel column (15 × 0.9 cm) equilibrated with Tris buffer containing 50 mM NaCl. The column was eluted with 50 ml of Tris

buffer followed by 400 ml of a linear gradient from 50 to 200 mM NaCl. Three-milliliter fractions were collected into 0.1 ml of Tris buffer containing 30 g/liter BSA.

This method has also been used to conjugate β-galactosidase to second antibodies raised against rabbit Fc fragments and guinea pig immunoglobulins.

Preparation of conjugates using MBS. Preparation of purified second antibody. Twenty milliliters of Sepharose–sheep IgG was mixed with 20 ml of donkey anti–sheep IgG antiserum (D11 4.1.78) for 1 h at room temperature, extensively washed with phosphate buffer, packed into a column, and eluted with 250 ml of 50 mM glycine–HCl buffer, pH 3.5, followed by 200 ml of 50 mM glycine–HCl buffer, pH 2.5. The eluant (4.5 ml fractions) was neutralized with either 0.5 ml of 0.2 M phosphate buffer, pH 7.4, or 0.5 M phosphate buffer, pH 8.0. The eluted material was pooled into two fractions, dialyzed extensively against distilled water at 4°C, and lyophilized. This lyophilized material still contained up to 40% inorganic salt which could be removed by dissolving the freeze-dried material in a minimum volume of distilled water and redialyzing. However, the presence of this concentration of salt did not affect the conjugation results so it was not normally removed. The purity of this material was verified by PAG electrophoresis.

Conjugation of enzyme to second antibodies using MBS. Purified donkey anti-sheep IgG antibodies (pH 2.5 fraction) were dissolved in 1.5 ml of 0.1 M phosphate buffer, pH 7.0, containing 50 mM NaCl to give an optical density of approximately 1.4 at 280 nm. A 15-μl aliquot of dioxan containing 0.32 mg of MBS was added to the antibody solution, mixed, and maintained at 30°C for 1 h. The solution was applied to a Sephadex G-25 column (30 × 0.9 cm), equilibrated with 10 mM phosphate buffer, pH 7.0, containing 10 mM $MgCl_2$ and 50 mM NaCl, and eluted with the same buffer. A total of 3 ml of eluant having an optical density of 0.70 (equivalent to a total protein content of 1.5 mg) was pooled.

One milliliter of phosphate buffer containing 1.5 mg of β-galactosidase was immediately mixed with the antibody eluted from the column and maintained at 30°C for 1 h. The reaction was terminated by the addition of 1 M mercaptoethanol to give a final concentration of 10 mM mercaptoethanol.

Purification of the conjugates. A 1.4-ml aliquot of the conjugate was applied to a DEAE-agarose column (15 × 0.9 cm) equilibrated with 10 mM Tris–HCl buffer, pH 7.0, containing 10 mM $MgCl_2$ and 10 mM mercaptoethanol. The column was eluted with 50 ml of the Tris buffer followed by 50 ml of Tris buffer containing 0.2 M NaCl. Three-milliliter fractions were collected into 0.1 ml of Tris buffer containing 30 g/liter BSA. The major peak of enzyme activity recovered from the column was pooled.

To determine the approximate extent of polymerization of the conjugate a 0.2-ml aliquot of the pooled material was chromatographed on a Sepharose 4B column (100 × 0.9 cm) equilibrated with Tris–HCl buffer and the elution profile of total and bound enzyme activity compared with that of untreated enzyme.

Detection of antibodies using enzyme–second antibody labels. The procedure for the detection of T_3 antibodies is described. Fifty micrograms of cellulose–T_3 solid phase in 0.1 ml of 50 mM barbitone buffer, pH 8.6, containing 1 g/liter BSA and 5 g/liter Tween 80 was mixed with 0.1 ml of diluted T_3 antiserum and 0.1 ml of barbitone buffer, pH 8.6, containing 1 g/liter BSA and left overnight at 4°C. The solid phase was washed once with 2 ml of barbitone buffer containing 50 g/liter NaCl and once with 2 ml of barbitone buffer containing 10 g/liter NaCl. A 0.1-ml aliquot of enzyme–antibody conjugate and 0.1 ml of barbitone buffer were mixed with the solid phase, left overnight at 4°C, washed as described above, and enzyme activity associated with the solid phase determined.

Before use the cellulose–T_3 solid phase

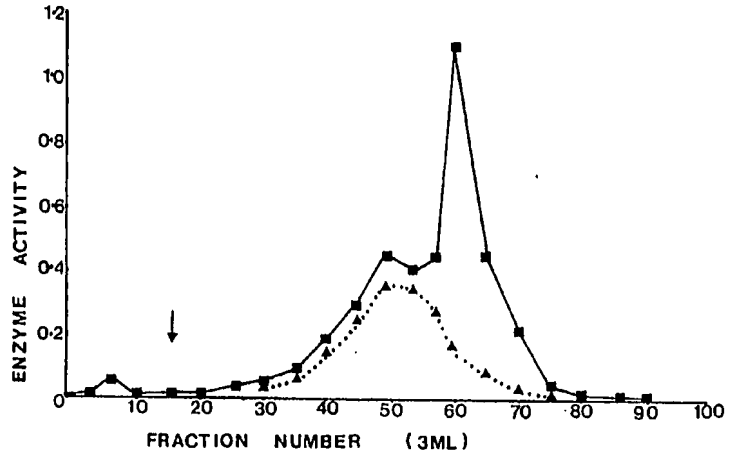

FIG. 1. Elution from DEAE-agarose of the enzyme-antibody conjugate prepared using *N,N'-o*-phenylenedimaleimide. ■, Total enzyme activity; ▲, bound enzyme activity. Enzyme activity is expressed as the increase in absorbance/30 min at 420 nm. ↓, Gradient applied.

was washed three times with 10 ml of barbitone buffer containing 5 g/liter Tween 80. The procedures for the detection of HgH, insulin, and cortisol antibodies were very similar to that described for the T_3 antibody except that the appropriate solid phase was used in each case.

RESULTS

Preparation of Conjugates using N,N'-o-Phenylenedimaleimide

An average of three sulfydryl residues were introduced into each antibody using mercaptobutyrimidate. Up to 80% of the enzyme was conjugated to immunologically active antibody with 90% retention of enzyme activity. The elution profile of total and bound enzyme activity from the DEAE-agarose is illustrated in Fig. 1. The bound enzyme was separated from nonconjugated antibody which eluted from the column before the gradient was applied, but was not completely separated from the remaining free enzyme by this purification system.

Preparation of Conjugates Using MBS

Typically 80% of the enzyme was conjugated to immunologically active antibody with approximately 90% retention of enzyme activity. The effect of conjugation on antibody activity is illustrated in Fig. 2. Approximately 10–15% of the antibody activity was lost during the coupling reaction. The elution profile of antibody and total and bound enzyme from the DEAE-agarose column is illustrated in Fig. 3. The purification procedure did not completely separate all the

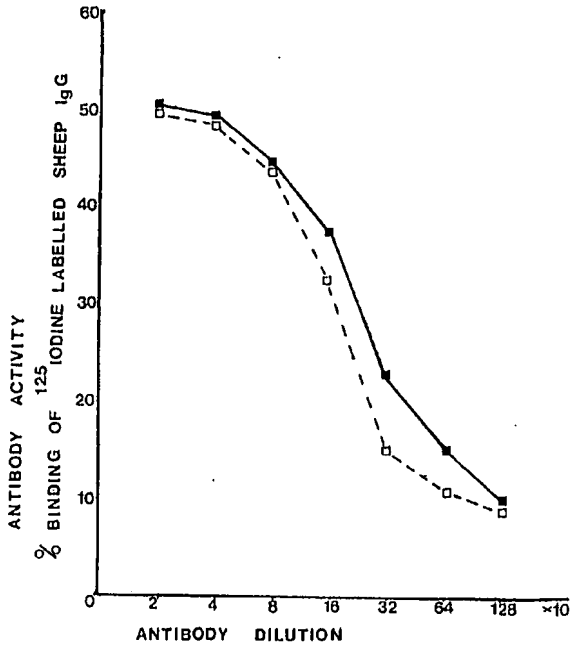

FIG. 2. Effect of conjugation on the activity of the second antibody. ■, Original antibody activity; □, activity remaining after coupling to the enzyme with MBS.

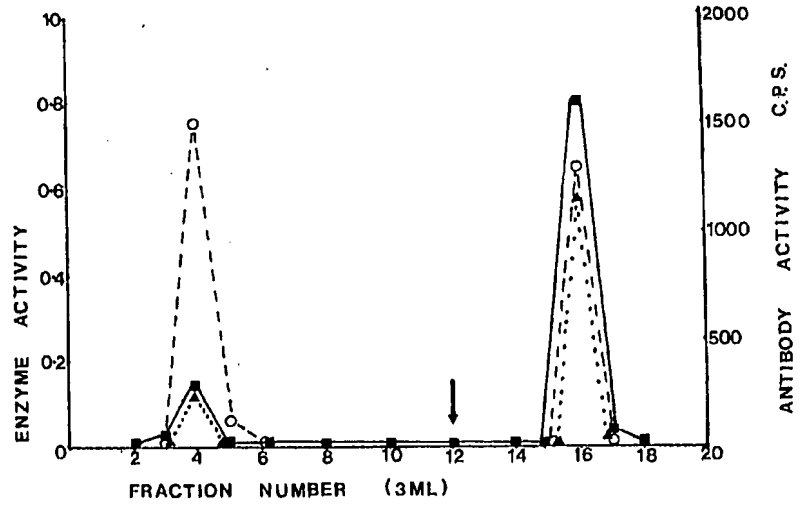

FIG. 3. Elution from DEAE-agarose of the enzyme–antibody conjugate prepared using MBS. ■, Total enzyme activity; ▲, bound enzyme activity. Enzyme activity is expressed as the increase in absorbance/30 min at 420 nm. ○, Antibody activity, binding of ^{125}I-labeled sheep IgG; ↓, 0.2 M NaCl applied.

conjugate from the free antibody. It is likely that a proportion of the enzyme is relatively heavily labeled with antibody and so chromatographs with the unlabeled antibody. No attempt was made to separate the conjugate from the remaining free enzyme.

The elution profile of this conjugate from Sepharose 4B is shown in Fig. 4. The majority of the conjugate (as measured by the bound enzyme activity) elutes only slightly ahead of the untreated enzyme, so the majority of the conjugate consists of nonpolymerized material. The coupling reaction was performed using a three- to fourfold molar excess of antibody over enzyme. Figure 3 illustrates that approximately 50% of the

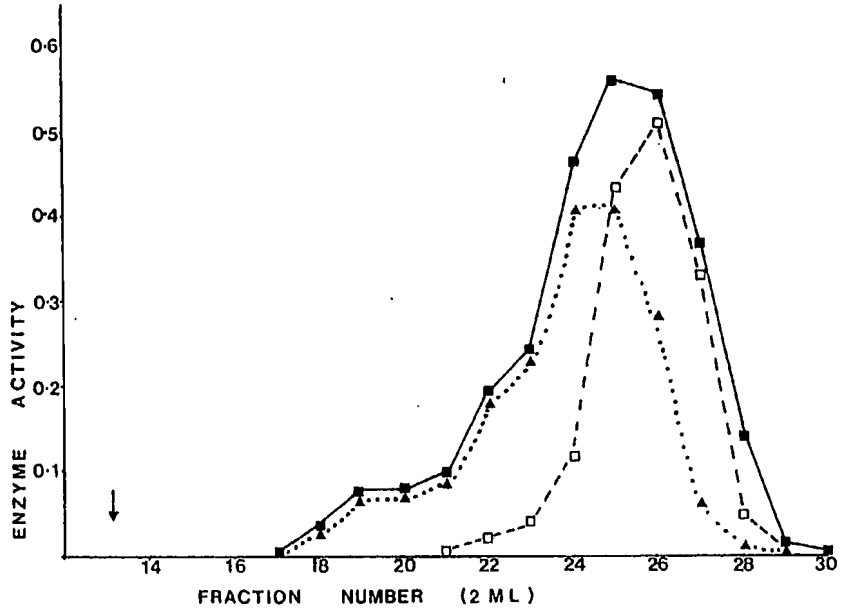

FIG. 4. Elution from Sepharose 4B of the enzyme–antibody conjugate prepared using MBS. Comparison with untreated enzyme. ■, Total enzyme activity of the conjugate; ▲, bound enzyme activity of the conjugate; □, activity of untreated enzyme. Enzyme activity is expressed as increase in absorbance/h at 420 nm. ↓, Void volume of the column.

antibody activity was coupled to enzyme. These results suggest that the conjugate contains about 1.5 to 2 antibody molecules per enzyme molecule.

Detection of Antibodies Using Enzyme–Second Antibody Labels

The detection of a number of different antibodies using the enzyme–antibody labels prepared using phenylenedimaleimide is illustrated in Fig. 5. The system can detect approximately 10 ng of T_3 antibodies, 5 ng of insulin antibodies, <30 ng of cortisol antibodies, and <5 ng of HgH antibodies. The sensitivity of the system is defined as that concentration of antibody which binds twice as much label as is bound in the absence of antibody.

The specificity of the reaction was checked in two ways:

(i) Binding of the label to the solid phase was very low in the presence of normal serum;

(ii) binding of the label was inhibited by the presence of free antigen in the system.

In the case of T_3 antibodies, binding was inhibited by the presence of T_3 but not by thyroxine (O'Sullivan, unpublished data).

Figure 6 shows a comparison between the ability of labels prepared using either phenylenedimaleimide or MBS to detect T_3 antibodies. The MBS label is slightly better at detecting low levels of T_3 antibodies.

Stability of the Conjugates

The β-galactosidase–antibody conjugate prepared using *N,N'-o*-phenylenedimaleimide retained 70% of its enzyme activity when stored in solution at 4°C in the presence of 0.02% (w/v) sodium azide for 1 year. No reduction in the proportion of bound enzyme activity occurred, indicating that both the crosslink and antibody are stable under these conditions. When stored for 2 months under the same conditions the β-galactosidase

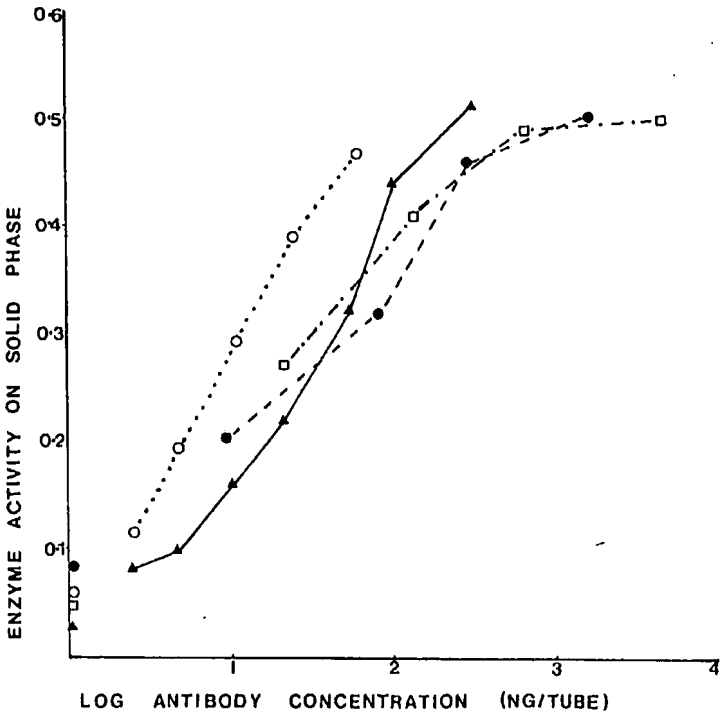

FIG. 5. Detection of antibodies using enzyme–second antibody conjugates. O, HgH antibodies; ▲, insulin antibodies; ●, T_3 antibodies; □, cortisol antibodies. Enzyme activity expressed as the increase in absorbance/h at 420 nm. Sufficient enzyme was added to produce an absorbance change of approximately 1.0/h for the total enzyme activity.

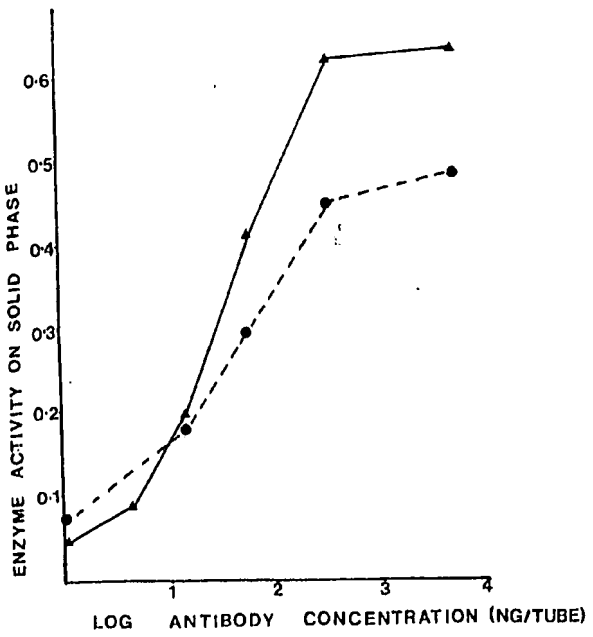

FIG. 6. Comparison of the labels prepared using the two conjugation methods for the detection of T_3 antibodies. ▲, Label prepared using MBS; ●, label prepared using *N,N'-o*-phenylenedimaleimide. Enzyme activity expressed as the increase in absorbance/h at 420 nm. Total enzyme activity added was sufficient to produce a change in absorbance of approximately 1.0/h.

antibody conjugate prepared using MBS retained 85% of its enzyme activity. No reduction in the proportion of bound enzyme activity occurred.

When the MBS label was frozen in the presence of 0.25% (w/v) BSA, no loss of enzyme activity occurred but the proportion of bound enzyme was reduced by approximately 10%. This label was stable for at least 2 months at −20°C. A similar situation occurred on lyophilization, with no reduction in total enzyme activity but the bound activity was reduced by 10%. The lyophilized label was stable for at least 2 months at −20 and +4°C but rapidly lost activity when stored at room temperature.

DISCUSSION

Phenylenedimaleimide will only react with antibodies containing free sulfydryl residues. Reduction with mercaptoethylamine (2) has previously been used to introduce such residues into antibodies. In this study, sulfydryl residues were introduced using mercaptobutyrimidate (17) which selectively modifies amino residues under mild conditions. In addition, this reagent does not alter the charge of the protein and so gross conformational changes in the antibody are likely to be avoided. Insertion of the sulfydryl residues was achieved while the antibodies were adsorbed onto an immunoadsorbent to minimize the possibility of damaging the antigen binding site. Following removal of excess reagent, the antibodies were eluted from the immunoadsorbent and reacted with the crosslinking reagent. As phenylenedimaleimide is a homobifunctional reagent the possibility exists that it can polymerize the antibodies. This possibility was reduced by adding a sufficiently large excess of reagent to saturate all the sulfydryl groups introduced into the antibodies.

With MBS there is no requirement to premodify the antibody. Furthermore, the heterobifunctional nature of MBS and the lack of free sulfydryl residues in antibodies ensures that there is no possibility of the reagent polymerizing the antibody molecules.

Using both methods a high proportion of the enzyme was conjugated to antibody with little loss in enzyme activity. Up to 80% of the enzyme was conjugated to antibody using phenylenedimaleimide and 80% using MBS. On several occasions using phenylenedimaleimide, precipitation occurred on addition of the enzyme to the antibody, which resulted in a reduced yield of soluble conjugate. This occurred more readily when using relatively high concentrations of enzyme and antibody.

Both labels could detect low levels of specific first antibodies, although the performance of the MBS label was slightly superior. A 10-fold increase in the assay sensitivity results from the use of a fluorimetric assay (18) for detecting β-galactosidase activity (O'Sullivan *et al.*, unpublished data).

The crosslink produced in both labels was highly stable, although with both labels

a slow loss of enzyme activity occurred on storage at 4°C. However, little or no loss of assay sensitivity occurred over the course of 1 year with the label prepared using *N,N'-o*-phenylenemaleimide.

The specificity of the detection of antigen-specific antibody by the labels was verified by the inhibition of label binding by free antigen. This has been used as the basis for a sensitive assay system to determine antigens. The method has the advantage that one label can be used to measure potentially any number of antigens. A preliminary communication of such a procedure has been published (19).

In conclusion, the results obtained using either a modification of the method introduced by Kato *et al.* (8) or the reagent MBS, which has not previously been used to prepare enzyme–antibody conjugates, appear to be preferable to methods more widely used to prepare such conjugates. Both reagents produced conjugates in high yield. The MBS method was simpler to perform and gave more reproducible results than the phenylenedimaleimide procedure.

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:
CHANG *et al.*
Appl. No.: 10/820,144
Filed: April 8, 2004
For: **Systemic Viral/Ligand Gene Delivery System and Gene Therapy**

Confirmation No.: 6653
Art Unit: 1632
Examiner: Shin-Lin Chen
Atty. Docket: 2474.0070003/BJD/JKM

Second Supplemental Information Disclosure Statement Under 37 C.F.R. § 1.97(c)

Mail Stop Amendment

Commissioner for Patents
PO Box 1450
Alexandria, VA 22313-1450

Sir:

Listed on accompanying IDS Forms, PTO/SB/08A and PTO/SB/08B, are documents that may be considered material to the examination of this application, in compliance with the duty of disclosure requirements of 37 C.F.R. §§ 1.56, 1.97 and 1.98. The numbering on this Second Supplemental Information Disclosure Statement is a continuation of the numbering in Applicants' First Supplemental Information Disclosure Statement filed on February 15, 2005 in connection with the above-captioned application.

Copies of documents **FP1** and **NPL1-NPL62** are submitted. However, in accordance with 37 C.F.R. § 1.98(a)(2), copies of U.S. patents, **US1-US10,** cited on the attached IDS Form, PTO/SB/08A, are not submitted.

Where the publication date of a listed document does not provide a month of publication, the year of publication of the listed document is sufficiently earlier than the effective U.S. filing date and any foreign priority date so that the month of publication is

not in issue. Applicants have listed publication dates on the attached IDS Forms based on information presently available to the undersigned. However, the listed publication dates should not be construed as an admission that the information was actually published on the date indicated.

Applicants reserve the right to establish the patentability of the claimed invention over any of the information provided herewith, and/or to prove that this information may not be prior art, and/or to prove that this information may not be enabling for the teachings purportedly offered.

This statement should not be construed as a representation that a search has been made, or that information more material to the examination of the present patent application does not exist. The Examiner is specifically requested not to rely solely on the material submitted herewith.

This Information Disclosure Statement is being filed more than three months after the U.S. filing date and after the mailing date of the first Office Action on the merits, but before the mailing date of a Final Rejection, or Notice of Allowance, or an action that otherwise closes prosecution in the application. Attached is our PTO-2038 Credit Payment Form in the amount of $180.00 in payment of the fee under 37 C.F.R. § 1.17(p).

It is respectfully requested that the Examiner initial and return a copy of the enclosed IDS Forms, and indicate in the official file wrapper of this patent application that the documents have been considered.

The U.S. Patent and Trademark Office is hereby authorized to charge any fee deficiency, or credit any overpayment, to our Deposit Account No. 19-0036.

Respectfully submitted,

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